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CONTRACT NUMBER DAMD17-96-C-6031

TITLE: Simple Semi-Quantitative Device for Hormones in Body Fluids

PRINCIPAL INVESTIGATOR: J. Michael Brinkley, Ph.D.
Mark Jackson, Gail Shannon

CONTRACTING ORGANIZATION: Emerald Diagnostics, Inc.
Eugene, Oregon 97402

REPORT DATE: October 1996

TYPE OF REPORT: Final, Phase I

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Final, Phase I (15 Mar 96-14 Sep 96)

4. TITLE AND SUBTITLE
Simple Semi-Quantitative Device for Hormones in Body Fluids

5. FUNDING NUMBERS
DAMD17-96-C-6031

6. AUTHOR(S)
J. Michael Brinkley, Ph.D. Mark Jackson
Gail Shannon

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Emerald Diagnostics, Inc.
Eugene, Oregon 97402

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

6 NOV 1996

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to DOD Components only (specific authority). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

Efforts to pinpoint phases of hormonal cycles through daily blood sample laboratory analyses have proven excessively expensive and impractical by routine laboratory methods. An attractive alternative is to employ immunochemical test strips to indicate critical ranges of key hormones and metabolites in easily obtained fluids such as urine since such techniques offer, in principle, rapid test results at modest cost. Such test strips require no instrumentation and minimal training of the user. During the Phase I studies, Emerald Diagnostics demonstrated technical feasibility of an enzyme amplified immunochromatography (EAIC) test strip platform for urine. This technology employed the enzymes glucose oxidase and horseradish peroxidase to generate color at the site of a specific antibody-mediated binding event. A sensitive competitive immunoassay was developed for the progesterone metabolite pregnanediol 3-glucuronide with a sensitivity of 1 μg/ml and a clinically useful dynamic range of 1 - 20 μg/ml. Feasibility was also demonstrated for a biotin/streptavidin model with sensitivity of 20 ng/ml of biotin and a fluorescein/anti-fluorescein model with sensitivity of 100 ng/ml of fluorescein. The EAIC technology was also able to detect 200 ng/ml of specific antibody in a whole serum sample matrix. Initial accelerated stability studies showed excellent stability of the EAIC components.

14. SUBJECT TERMS
Enzyme Amplified Immunochromatography
Competitive Immunoassay
Hormone detection

15. NUMBER OF PAGES
31

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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1. TABLE OF CONTENTS

2. INTRODUCTION

2.1. SUBJECT AND GENERAL BACKGROUND

Progesterone is secreted by the corpus luteum in the post-ovulatory phase of the menstrual cycle. Progesterone is metabolized and excreted in urine as pregnanediol 3-glucuronide (PDG). The results from numerous studies of ovarian function and steroid metabolism have suggested that the principal urinary metabolites of estradiol and progesterone might be used to identify the fertile period in women [1]. A 1982 study conducted by the World Health Organization [1] found that the measurement of estrogen metabolite estrone-3-glucuronide (E3G) and PDG could be used to indicate the start and end of the fertile period in over 80% of the ovarian cycles studied. However, subsequent efforts to pinpoint phases of hormonal cycles through daily blood sample analyses have proven excessively expensive and impractical by routine laboratory methods. An attractive alternative is to employ immunochemical test strips to indicate critical ranges of key hormones and metabolites in blood or urine since such techniques offer rapid test results with minimum sample preparation at modest cost.

The availability of such a test to identify the start and duration of each fertile period would be of value to couples who wish to practice family planning by periodic abstinence, or limit the use of barrier methods for contraception to this phase of the menstrual cycle. Conversely, the same information can be used to improve the timing of intercourse by women who desire to become pregnant. In particular a sign of impending ovulation would be of value in the treatment of patients who are infertile as a result of ovarian dysfunction or for the purpose of artificial insemination [1].

2.2. DESCRIPTION OF METHOD

The proposed immunochemical test method is an improvement on the immunochromatography technique now in commercial use in home pregnancy tests and similar devices. These devices are generically termed lateral flow devices. The disposable test device typically

consists of a plastic holder containing an absorbent material that protrudes on one side. Localized on this absorbent are intensely colored particles that carry an antigen or antibody on the particle surface. For the sake of example, assume the particles are treated with antigen. These surface modified particles are localized on the absorbent near the protruding area. When the protruding material is placed in a sample of water or biological fluid, liquid is drawn into the absorbent. This flow of liquid causes the particles to move along the absorbent. A line of deposited immobilized antibody to the antigen is next encountered by the advancing particles. If the sample contains no analyte, the particles are trapped by the immobilized antibody. The plastic device may hide particles trapped in this region from view. If free antigen is found in the sample, some antibody is used to bind this antigen and the antibody is then not available to bind the particles. These particles then break through the line of deposited antibody. The particles continue to migrate to a second area of deposited antibody that traps them. This second line of antibody is separated spatially from the first. Deposited particles can be seen in this area by the design of the holder. The appearance of the colored particles in this region is a positive test result.

For analyses requiring very high sensitivity, the use of visually detected colored particles is inherently inadequate. Bangs [2] has estimated that latex particle agglutination in the size range of 0.1 μm will detect about 10^{-10} moles of material with a molecular weight of 100,000. With the molecular weight of progesterone at 496.6 and a desired limit of detection of 10 ppb, the concentration of progesterone is $5x10^{-11}$ moles/liter. That is, simple agglutination methods will be barely able to detect the desired level of material. The immunochromatographic methods are less well studied than agglutination methods but we do not expect them to be more sensitive than agglutination, just easier to read. Therefore, we believe that an amplification method must be found which will enhance the sensitivity by a reasonably large factor (say 10 to 100) over simple visual agglutination detection. We have found a way to amplify the immunochromatography technique without loss of the ease of use features.

Enzyme amplified immunochromatography (EAIC) involves a device with the same external appearances as the normal immunochromatographic device. In this case, however, the particles are coated with the antigen plus the enzyme peroxidase, although many variants are possible. The enzyme catalyzed reactions used are the following:

$$\text{glucose} + \text{oxygen} \xrightarrow{\text{glucose oxidase (GO)}} H_2O_2 + \text{glucuronic acid}$$

$$H_2O_2 + \underset{\text{(uncolored)}}{\text{tetramethylbenzidine}} \xrightarrow{\text{peroxidase (HRP)}} \text{blue to brown colored dye}$$

These are the familiar color-forming reactions that have been tested for years in dipsticks for measuring glucose in blood and urine. The diabetes test strip technology has used this system for decades. Thus, stability and other properties of the system in absorbent matrices of the type proposed here are generally known.

The major problem with an enzyme amplified immunochromatographic method is the need to isolate the enzyme reactions spatially. The substrates for the reaction, unlike the proteins, cannot be immobilized. Products of the enzymatic reaction can diffuse from one region to another thereby producing false positives or at least causing high background color. In the

case of glucose oxidase and similar enzymes, however, there is a simple way to localize color generation spatially based on two properties of the system. First, the use of tetramethyl-benzidine as the indicator produces a water insoluble colored product that will not diffuse. Thus, the final color is fixed in place. Second, reaction zones can be separated from each other by immobilized catalase. This will destroy any hydrogen peroxide formed outside of the viewing region before it can interfere.

2.3. *Merits of Lateral Flow Immunochromatographic Test Strip Platform*

Immunochromatographic test strips as a generic assay platform afford considerable promise in the Army's search for simple cost-effective means to analyze body fluid samples for hormones of choice. These include:

- Ease of use with the potential adaptation of the device for various body fluid types, such as urine, saliva, whole blood or blood components, such as serum
- No requirements for instrumentation in order to obtain test result
- Minimal requirements for special training of personnel in use of device
- Convenient packaging to provide for small number of tests at modest cost
- Significant shelf life of a year or more
- Sensitivity which can be adjusted to suit the assay need
- Potential amplification of analyte detection signal through enzymatic methods as developed in this Phase I study
- Qualitative or semi-quantitative test results
- Multiple analyte capability

2.4. *Phase I Scope and Technical Objectives*

Emerald Diagnostics' Phase I studies aimed to demonstrate feasibility and sensitivity of a semi-quantitative test strip that used the enzyme amplified immunochromatography technology. Feasibility was demonstrated with a single analyte device for the semi-quantitative measurement of metabolized progesterone (PDG) in urine. Studies demonstrated the lower limit of sensitivity for the proprietary techniques for amplifying the detection system. It was anticipated that the format would provide a measurement range of concentration 10-100 fold above a lower sensitivity limit which can be adjusted to suit the diagnostic problem. The stability of the device was assessed based upon accelerated stress studies.

The following technical objectives were targeted for the Phase I project:

1. Develop a prototype test device that will measure levels of pregnanediol 3-glucuronide in urine in the low ng/ml range
2. The device will permit the semi-quantitative estimate of higher concentrations
3. Target shelf-life of 18 - 24 months - based upon accelerated stability studies
4. Develop a prototype modification of the device that will allow the use of serum or plasma samples

2.5. TERMINOLOGY AND ABBREVIATIONS

RabIgG; *Rabbit Immunoglobulin IgG*, Gt-X-RabIgG; *goat anti-rabbit immunoglobulin G*, HRP; *horseradish peroxidase*, GO; *glucose oxidase*, TMB; *3, 3', 5, 5'-tetramethyl-benzidine*, H_2O_2; *hydrogen peroxide*, DMSO; *dimethyl sulfoxide*, BSA; *bovine serum albumin*, PDG; *pregnanediol 3-glucuronide*, EAIC; *Enzyme amplified immuno-chromatography*, FITC; *fluorescein isothiocyanate*, E3G; *estrone-3-glucuronide*, TBS; *Tris buffered saline*, NFDM; *non-fat dry milk*, DI; *reagent grade deionized water*, RT; *room temperature*, BCA; *Bicinchoninic acid.*

2.6. MATERIALS

2.6.1. Test Device

Millipore nitrocellulose membrane: 15 μm SRHF04000, 20 μm STHF04000, 25 μm SXHF04000
Gelman Conjugate Pads: S70011
Schleicher and Schuell Absorbent Pad: Grade 470 Paper
Adhesive Research double-sided tape: Arcare 7841CO#1513H

2.6.2. Buffers

50 mM phosphate, 2.5% BSA, 2% Dextran 40,000 molecular weight (MW), 0.05% Tween 20, pH 7.2 **(PB/BSA)**
50 mM phosphate, 2.5% BSA, 2% Dextran 40,000 molecular weight (MW), 0.05% Tween 20, 1% glucose, pH 7.2 **(PB/BSA-glucose)**

2.6.3. Immunoreagents

RabIgG: Zymed 02-6102
Gt-X-RabIgG-HRP: Chemicon AP132P
Ms anti-PDG Monoclonal: Serotec MCA399, Crystal Chem P-10E, Fitzgerald 10-P67, ICN 63-353
PDG: Sigma P-3635
Streptavidin: Pierce 21125B
Biotin: Lancaster Synthesis 5109
Biotinylated BSA: Pierce 29130
Streptavidin-HRP: Kirkegaard & Perry Laboratories (KPL) 14-30-00
Biotinylated HRP: Pierce 29139
TMB Microwell Peroxidase Substrate: KPL 50-76-05
TMB Membrane Peroxidase Substrate: KPL 50-77-03
TMB: Aldrich 86,033-6
Glucose Oxidase: Sigma G-6891
Catalase: Sigma C3515
Mouse anti-fluorescein isothiocyanate (FITC): Zymed 13-9600
FITC-Peroxidase: Sigma P-2649
FITC: J.T. Baker M422
Human IgG: Sigma I-4506
Mouse anti-human IgG: I-6760

Goat anti-mouse IgG-HRP 62-6520
Mouse serum: Sigma M-5905

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1. GENERAL PROCEDURES

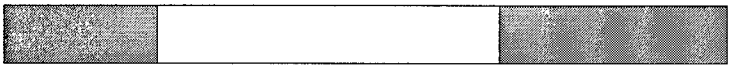
3.1.1. Construction of Test Device

The test device consisted of the following components. Nitrocellulose membrane from Millipore Membrane was used as the reaction solid phase to which antibody was applied. Antibody was applied either by micropipetter (spotted) or by use of an airbrush applicator system (Biodot, Irvine CA). Nitrocellulose membranes of various pore sizes (as specified in the materials list) were tested. Pore size is directly related to flow rates; with larger pore sizes giving faster flow rates. A 5 X 25 mm strip of membrane was attached to a plastic holder by use of double-sided adhesive tape specifically used in lateral flow devices.

To the bottom end of the membrane, a 5 X 20 mm piece of glass fiber material was attached via the adhesive tape. The glass fiber pad overlapped onto the membrane by approximately 2 mm. Enzyme labeled antigen was applied and dried onto the glass fiber pad. Sample was also added to the pad to begin running the test.

An absorbent paper was attached to the top end of the membrane with an approximate overlap of 2 mm. This pad absorbed the sample as it migrated through the membrane. The final configuration of the test device is depicted in Figure 1.

Figure 1: Immunochromatography Test Strip



Glass Fiber Pad Nitrocellulose Membrane Absorbent Pad

3.1.2. Use of Model Systems

For many of the experiments, especially with the component studies, a model system employing RabIgG as the antigen and the antibody, Gt-X-RabIgG, was used. This was done for two reasons. First, these reagents were known to work in multiple immunoassay formats (ELISA, Western Blots, etc.) and were available in high purity with high binding affinities. This allowed the assay format components to be evaluated in a known working system before introducing antigen/antibody reagents that were less well characterized in assay performance. Secondly, the PDG antibodies and antigens were costly. We felt the use of PDG specific reagents was not necessary to understand how the individual components performed in the system and that the results were "generic" in nature to any antigen-antibody model used.

Further into the project, other model systems were used to identify the lower limits of sensitivity of the EAIC technology when applied to various types of analyte measurements. These models included streptavidin-biotin and FITC with anti-FITC antibodies.

3.1.3. Streptavidin-Biotin

Streptavidin is a 60,000 dalton protein isolated from *Streptomyces avidinii*. Biotin is a 244 dalton vitamin that binds with high affinity to streptavidin. These reagents have been developed and used in several immunoassay formats to amplify and increase the sensitivity of an assay. This increase comes from the high affinity these reagents have for each other and the rapid rate at which the binding occurs.

These reagents were selected for use in the development of the PDG assay for several reasons. First, we used streptavidin as the carrier protein to couple the PDG hormone which then allowed us to attach the enzyme by use of biotinylated HRP. We also used biotinylated BSA as the carrier protein and streptavidin labeled with HRP. These two combinations gave us the flexibility to link the antigen and the enzyme together without having to use particles as the "carriers". Instead, the antigen and enzyme were linked directly together via the streptavidin and biotin. In addition, by using the streptavidin and biotin we were able to take advantage of the increased assay sensitivity possible with this system.

3.2. COMPONENT STUDIES

Studies were performed on the components in the EAIC technology. These components included glucose oxidase, catalase and TMB. The experiments were performed to gain an understanding of the individual components before they were combined in use. The studies focused on identifying the concentrations required for each component in its final intended use. In addition, stability studies were performed for the glucose oxidase and TMB substrate.

3.2.1. Glucose Oxidase Studies -Concentration Requirements

Experiments were conducted to determine the required concentration of glucose oxidase to be applied to the membrane in order to generate the following reaction:

$$\text{glucose} + O_2 \xrightarrow{\text{GO}} H_2O_2 + \text{glucuronic acid}$$

$$H_2O_2 + \text{TMB} \xrightarrow{\text{HRP}} \text{Blue to brown dye}$$

Two experiments were performed. The first one was done using an HRP latex conjugate and a second one was run using the biotin-streptavidin model without latex particles. The concentration was determined to be adequate if a blue/green reaction color was generated within 5 minutes.

Materials and Methods

In the first experiment, commercially prepared TMB from Kirkegaard & Perry Laboratories (KPL) was applied to Millipore ST membrane as 5 µl spots and dried for 2.5 hr. to remove the H_2O_2 present in the TMB solution. Glucose oxidase (GO) enzyme at 1200 units/ml was diluted in Tris buffered saline (TBS) and 5 µl applied and dried on the membrane at the site of the TMB. Gt-X-Rab-HRP latex conjugate was diluted 1/8 in TBS containing 1% glucose and 5 µl

was applied to the TMB/GO site on the membrane. The time required for a blue/green reaction color to develop was determined.

For the second experiment, 4 µl of streptavidin was applied to Millipore SR membrane at 1 mg/ml. TMB substrate solution was prepared by dissolving 10 mg of Aldrich TMB in 1 ml of DMSO. This TMB stock solution was diluted to 1 mg/ml in DI water + 10% ethanol that also contained various concentrations of glucose oxidase (12-120 units/ml). Five microliters of this substrate solution was applied to the streptavidin location on the membrane and dried for 20 minutes.

Biotinylated HRP was diluted 1/1000 in PB/BSA-glucose buffer. This reaction mixture was migrated through the membrane (150 µl) and color development noted. Results

Results from the first experiment shown in Table 1 indicated that GO concentrations of 12 - 1200 units/ml were adequate in generating acceptable TMB reaction color very quickly. Without GO, no reaction was seen.

Table 1: Glucose Oxidase Concentration vs. Time for Color Reaction

GO Concentration	Time for Color Reaction
0 units/ml	Negative - no color reaction
12 units/ml	+ @ 0.5 min.
120 units/ml	+ @ 0.5 min.
240 units/ml	+ @ 0.5 min.
1200 units/ml	+ @ 0.5 min.

Results for the second experiment (Table 2) demonstrated that GO concentrations of 12-120 units/ml generated adequate color, however the signal was slightly lighter for 12 units/ml.

Table 2: Glucose Oxidase Concentration vs. Color Reaction

GO Concentration	Color
12 units/ml	+ (lighter)
60 units/ml	+
120 units/ml	+

3.2.2. Inhibition of TMB Reaction by Use of Catalase

Catalase was applied to the membrane in order to destroy $H_2O_2$and inhibit the TMB reaction. Catalase could be used to isolate reaction areas and prevent the formation of color from diffused substrate. The inhibition of the TMB reaction color at the site of TMB, HRP latex conjugate and H_2O_2 was used to determine the activity of the catalase enzyme.

Materials and Methods

TMB prepared by KPL (2.5 µl) was applied to Millipore SX membrane and dried for 2.5 hours. Catalase diluted in TBS was applied to the membrane at a location below the TMB. Gt-X-Rab-HRP latex conjugate at 4.2% solids was diluted 1/10 in TBS, 30% sucrose and 2% NFDM and

20 µl was dried onto glass fiber pads. TBS containing 0.006% H_2O_2 was added to the pads (100 µl) to migrate the HRP conjugate through the membrane. A positive control was run using TMB membrane without catalase. A negative control was run using TBS without H_2O_2.

Another experiment was performed to determine if the HRP conjugate could migrate past the catalase on the membrane and still remain active. Rab IgG (µ) was applied at two locations on Millipore SX membrane. Five microliters of TMB (KPL) + 240 units/ml of glucose oxidase (GO) were also applied and dried onto the same location on the membrane. Catalase at 7788 units/ml in TBS (2.5 µl) was applied to the membrane between the two RabIgG/GO/TMB locations. Glass fiber pads were prepared with 25 µl of Gt-X-Rab-HRP latex conjugate at 4.2% solids diluted 1/5 in TBS, 30% sucrose and 2% NFDM. TBS + 1% glucose (100 µl) was used to migrate the HRP conjugate through the membrane.

Results

Results in Table 3 show that catalase was able to inhibit the TMB reaction by the destruction of H_2O_2. A concentration of approximately 7788 units/ml catalase was required to destroy 0.006% H_2O_2. Lower concentrations of catalase did not inhibit the TMB reaction. Catalase concentration requirements could change based upon the amount of H_2O_2 present.

Table 3: Formation of TMB Reaction Color in the Presence of Catalase

Test Condition	TMB Reaction Color
Positive Control - 0 units/ml	+ @ 1 min.
78 units/ml	+ @ 0.5 min.
779 units/ml	+ @ 1 min.
7788 units/ml	negative
Negative Control - no H_2O_2	negative

In the second experiment, the HRP conjugate was able to generate color at both TMB/GO locations on the membrane which proved that the HRP reagent could migrate past the catalase and still remain active.

3.2.3. Accelerated Stability Studies - TMB and Glucose Oxidase

An experiment to determine the initial accelerated stability profile for TMB and glucose oxidase dried onto membrane material was performed. Previous experience with conducting stability studies for this type of immunoassay has shown that 10 days at the accelerated temperature of 45°C is equivalent to approximately 12 months at room temperature (RT). This stability criterion was selected as a target for the initial stability study and component stability was monitored for 7 to 11 days at 45°C.

Materials and Methods

For this study, the TMB was applied to the membrane by use of the Biodot airbrush applicator. TMB from KPL was not stable on the membrane after airbrush application. The instability was seen as oxidation of the substrate and the development of blue color on the membrane at the location of the TMB. For this reason a different substrate formulation was tested. TMB from Aldrich was prepared as a 20 mg/ml stock in DMSO. The TMB was then diluted to 1 mg/ml in DI water + 10% ethanol. This solution was applied to Millipore ST membrane by airbrush

membrane were then sealed in a foil pouch containing 1 g of molecular sieve desiccant and placed at RT and 45°C.

Stability time points were run at day 3 and day 7. The membrane was tested for TMB activity by applying 5 µl of Gt-X-RabIgG-HRP conjugate diluted 1/200 in 10 mM Tris buffer +0.006% H_2O_2.

For the glucose oxidase stability study Millipore ST membrane was prepared with 5µl spots of glucose oxidase at 1200 units/ml. The strips were dried at RT for 20 min. and then sealed in foil pouches containing 1 gram of molecular sieve desiccant. Pouches were stored at RT and 45°C. Several time points were tested over 11 days and fresh strips were prepared at each time point to serve as the positive control. Before testing, 5 µl of TMB (KPL) was added to the membrane at the site of the glucose oxidase and dried for 2.5 hr. Gt-X-Rab-HRP latex conjugate diluted 1/8 in TBS + 1% glucose was then added to the membrane. The time required for a positive blue reaction color was recorded.

Results

The TMB stability results are listed in Table 4. The performance after 7 days at the accelerated temperature was still acceptable. The light green line seen before testing was due to some oxidation of the substrate on the membrane. Whether this was due to true lack of stability or an artifact of the substrate being placed at such a high temperature will need to be determined by conducting real time stability studies at a future date.

Table 4: TMB Accelerated Stability Results

Stability Time Point	Color Reaction
Positive Control (Fresh)	dark blue
Day 3 - RT	dark blue
Day 7 - RT	dark green (@ 1 min.) dark blue (@ 5 min.)
Day 3 - 45°C	dark blue
Day 7 - 45°C	light green before testing dark green @ 1 min.

Table 5 lists the positive reaction time for the glucose oxidase membranes placed on accelerated stability. The results indicated that the enzyme lost no measurable activity after 11 days at 45°C.

Table 5: Glucose Oxidase Accelerated Stability Study

Time Point	Positive Control	RT	45°C
Day 2	+ @ 1 min.	+ @ 1 min.	+ @ 1 min.
Day 4	+ @ 1 min.	+ @ 1 min.	+ @ 1 min.
Day 7	+ @ 1 min.	+ @ 1 min.	+ @ 1 min.
Day 11	+ @ 1 min.	+ @ 1 min.	+ @ 1 min.

Discussion on Component Studies

The results of the component studies determined the feasibility of using several critical components of the EAIC technology. We were able to demonstrate the use of glucose oxidase combined with the use of peroxidase to generate a positive color reaction with TMB substrate. We also inhibited this activity by the use of catalase which destroyed the H_2O_2 in the system. Reagents were able to migrate past the catalase and still remain active.

Stability studies of the TMB substrate and glucose oxidase enzyme indicated that both of the components have good stability at accelerated temperature which should translate to long shelf life.

There are solubility problems with the Aldrich TMB and the formulation will need to be optimized to increase the solubility of the substrate.

3.3. DEVELOPMENT OF PDG COMPETITIVE ASSAY

The PDG assay was developed by conjugating the antigen to commercially prepared streptavidin or biotinylated BSA. These antigen preparations then were combined with either commercially prepared biotinylated HRP or streptavidin HRP. After an incubation period, the PDG antigen and HRP were linked together by the streptavidin-biotin, resulting in a PDG/HRP conjugate complex.

Several commercially available anti-PDG antibodies were evaluated by ELISA and lateral flow formats using the PDG antigen preparations described above. The clone that performed the best on lateral flow was selected for use in the feasibility studies.

Most of the studies were performed in a buffer system. The use of urine as the sample matrix was also examined.

3.3.1. Evaluation of Commercially Available PDG Antibodies

Both polyclonal and monoclonal antibodies against PDG were commercially available. All of the polyclonals were supplied as antisera. Those posed a purity problem, since antibody applied to membrane for lateral flow tests needs to be in very pure form so that a high amount of specific antibody can be applied to the membrane. In order to purify the anti-PDG antisera, it would have been necessary to make a PDG column. We decided not to undertake this process at this time because of the time and expense required. Instead, we evaluated 4 commercially available anti-PDG monoclonal antibodies. One was purified and the other three were easily purified using a Pierce Protein A purification kit (44667). This kit contained prepared affinity columns, desalting columns and buffers which made the purification step rapid and efficient.

The anti-PDG monoclonal antibodies were evaluated in ELISA and lateral flow formats. However, performance in the lateral flow was the deciding factor since this was the format of the final test. ELISA was performed for informational purposes only.

Materials and Methods

The following commercial anti-PDG monoclonal antibodies were evaluated: Crystal Chem (clone # P-10E), Fitzgerald (clone # 10-P67), ICN (clone # P44), Serotec (clone # B012). The Crystal Chem was available in purified form and the Fitzgerald, ICN and Serotec were purified by Protein A.

The ELISA was performed by coating 2 μg/ml antibody in PBS, 50 μl/well, overnight at 4°C. The wells were washed 2X using 10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.2. After washing, the wells were blocked with TBS containing 2% non-fat dry milk (Block Buffer), 100 μl/well, for 1 hour at RT. The block solution was then aspirated from the wells without washing. PDG antigen coupled to biotinylated BSA and PDG coupled to streptavidin were diluted in block buffer 1/10, 1/100, 1/1000 and 1/10,000. The antigen dilutions were added to the antibody coated wells, 50 μl/well and incubated for 1.5 hr. at RT. The wells were then washed 2X to remove unbound material. Streptavidin-HRP at 1/1000 was added to the wells containing the PDG:biotinylated BSA antigen and biotinylated HRP was added to the wells containing the PDG:streptavidin antigen. All wells received 50 μl of conjugate and were incubated for 1 hour at RT. After a 3X wash, TMB (KPL) substrate was added to the wells, 50 μl/well and color developed for 10 min. before the reaction was stopped by the addition of 25 μl of 0.18M sulfuric acid. Absorbance of the wells was read at 450 nm using a standard microwell plate reader.

The lateral flow testing was performed by spotting the antibodies at 1 mg/ml onto Millipore SR membrane, 2.5μl per spot. After drying for 30 min., the membrane was prepared for testing.

PDG-biotinylated BSA was diluted 1/10,000 with streptavidin-HRP added to the dilution at 1/5000. PDG-streptavidin was diluted 1/10,000 with biotinylated HRP added to the dilution at 1/5000. These mixtures were incubated at RT for 45 min. to allow the streptavidin and biotin to bind together.

After the antigens were incubated, 150 μl of the solutions were migrated through the membrane for 20 min. Negative controls were performed by using 150 μl of the HRP dilutions at 1/5000. This was done to check for non-specific binding of the HRP conjugates to the antibody on the membrane. TMB (KPL) was then added to the membrane (50μl) and color development noted.

Results and Discussion

The ELISA results are shown in Figure 2 for the PDG-streptavidin antigen preparation. Results were similar for the biotinylated BSA-PDG antigen. Three of the four monoclonals captured the PDG antigen with Serotec giving the strongest response. The lack of capture by the ICN antibody was surprising; however it could be due to the antibody epitope being destroyed when the PDG molecule was coupled to the carrier proteins or due to poor integrity of the antibody preparation.

The results for the lateral flow testing are listed in Table 6. Again 3 of the 4 antibodies captured the PDG antigen preparations, with Serotec giving the best performance and ICN not capturing at all. However, unlike the ELISA, the Fitzgerald antibody did not perform as well and no capture was seen with the PDG-streptavidin preparation. The HRP conjugates alone did not generate any signal which indicated that specific binding occurred between the antibodies and the antigens.

Figure 2.

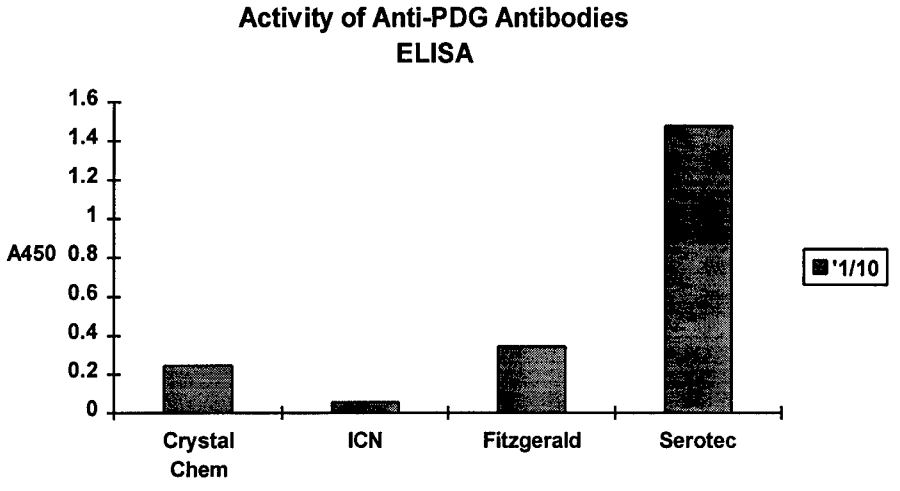


Table 6. Performance of Anti-PDG Antibodies in Lateral Flow Format

PDG Antigen	HRP	Crystal Chem	Fitzgerald	ICN	Serotec
none	SA-HRP	-	-	-	-
none	Bio-HRP	-	-	-	-
Biotinylated BSA-PDG	SA-HRP	+	+/-	-	+
Streptavidin-PDG	Bio-HRP	+	-	-	+

3.3.2. Preparation and Evaluation of PDG:biotinylated BSA and PDG:streptavidin Conjugates

PDG was conjugated to the carrier proteins biotinylated BSA and streptavidin. This allowed for the use of streptavidin-HRP or biotinylated HRP, respectively, to link the PDG and HRP together for the competitive assay. The conjugation was determined to be successful if PDG was conjugated to the biotinylated BSA and streptavidin without destroying the antibody binding epitope on the PDG molecule and the biotin or streptavidin binding activity. Activity was tested by ELISA and lateral flow formats.

3.3.2.1. Preparation of PDG:biotinylated BSA and PDG:streptavidin Conjugates

The PDG was activated by dissolving 9 mg. of PDG into 400µl of dry dimethyl formamide with constant stirring in an ice bath under nitrogen. Diisopropylethylamine and isobutylchlorocarbonate (3.1 µl and 2.3 µl respectively) were added and allowed to react for 20 min.

The activated PDG was conjugated to both biotinylated BSA and streptavidin at a molar ratio of 100:1. The activated PDG (3.8 mg.) was added drop-wise to 1 ml of 5 mg/ml streptavidin in 100 mM carbonate buffer pH 9.5. To a 1 ml solution of 5 mg/ml biotinylated BSA also in 100 mM carbonate was added 4.02 mg. of the activated PDG. Both solutions were allowed to react for 4 hours.

After 4 hours, the reaction systems were dialyzed in 50 mM phosphate buffer, pH 7.4 overnight. After dialysis the solutions were centrifuged and applied to G-50 columns to separate the unconjugated PDG from the conjugated species. Fractions were checked for absorbance at 280 nm and protein containing fractions were pooled. Protein concentration was determined by the Bicinchoninic acid (BCA) protein assay (Pierce).

3.3.2.2. Determination of PDG and Biotin/Streptavidin Activity for Antigen Conjugate - ELISA

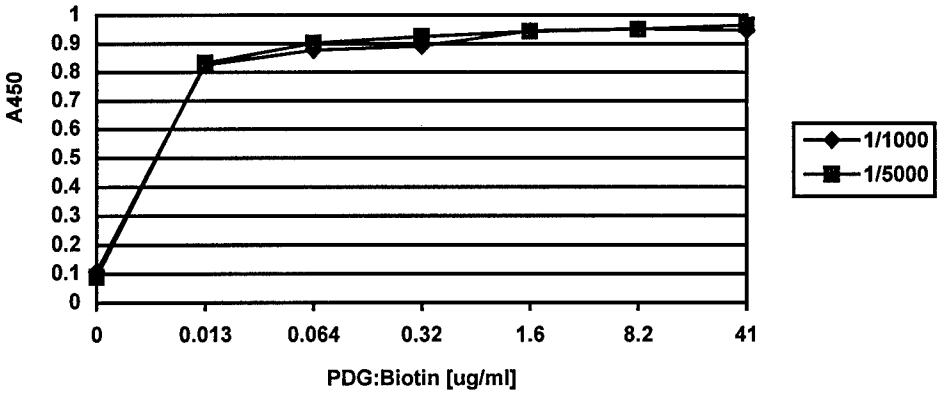
Materials and Methods

Serotec anti-PDG antibody was coated at 2 µg/ml in phosphate buffered saline (PBS) onto microtiter wells (50 µl/well) overnight at 4°C. The wells were washed 2X using 10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.2. After washing, the wells were blocked with 100 µl of 10 mM Tris buffer containing 2% non-fat dry milk (Block Buffer) for 1 hr. at RT. After blocking, the wells were aspirated to remove the block solution. The PDG:biotinylated BSA at 410 µg/ml and the PDG:streptavidin at 630 µg/ml were diluted 1/10 - 1/31,250 (1/5 serial dilutions) into block buffer and added to the antibody coated wells (50 µl/well). The antigen preparations were incubated in the wells for 2.5 hr. RT. After washing 2X to remove unbound material, streptavidin-HRP diluted 1/1000 and 1/5000 in block buffer was added to the wells that contained the PDG:biotinylated BSA. Biotinylated-HRP at 1/1000 and 1/5000 dilution was added to the PDG:streptavidin wells. All the wells received 50 µl of conjugate and were incubated for 1 hr. at RT. After washing 3X, 50 µl of TMB substrate (KPL) was added and allowed to react for 10 min. before being stopped by the addition of 25 µl of 0.18M sulfuric acid. Absorbance of the wells was determined at 450 nm.

Results and Discussion

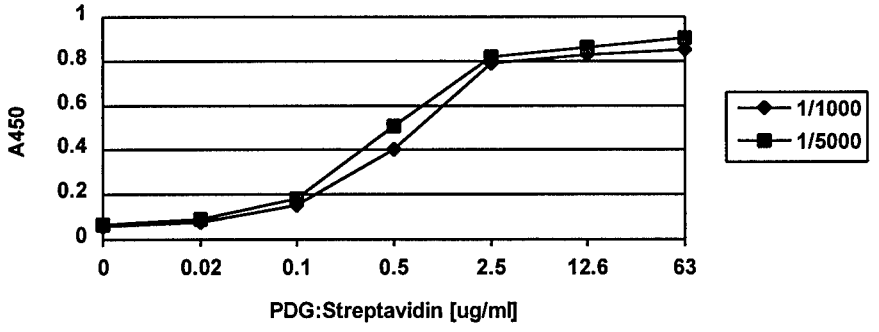
Absorbance results for the PDG:biotinylated BSA preparation are shown in Graph 1. The assay signal was very high for all dilutions tested and this indicated that the PDG and biotin activity were still present after the conjugation procedure.

Graph 1: Activity of PDG:Biotinylated BSA



The assay signals generated from the PDG:streptavidin antigen preparation are shown in Graph 2. Again, the PDG and streptavidin activities were very high.

Graph 2: Activity of PDG:Streptavidin



These results indicated that PDG can be successfully conjugated to biotinylated BSA and streptavidin and both the PDG and biotin-streptavidin activities remain intact.

3.3.2.3. Determination of PDG and Biotin-Streptavidin Activity for Antigen Conjugates - Lateral Flow

Materials and Methods

Serotec antibody at 0.5 mg/ml in PBS was spotted (5 µl) onto Millipore ST membrane. Various dilutions of the PDG:biotinylated BSA were combined with various dilutions of streptavidin-HRP using PB/BSA as the diluent. PDG:streptavidin dilutions were mixed with biotinylated-HRP. These antigen-HRP mixtures were incubated for 45 min. to allow the biotin and streptavidin to bind together. A small amount (30 µl) of the reaction mixtures was then migrated through the

Serotec anti-PDG membrane for 10 min. A negative control of streptavidin-HRP or biotinylated-HRP was run to check the background signal of the HRP conjugate. TMB from KPL (50μl) was added to the membrane to indicate the presence of HRP.

Results and Discussion

Results for the PDG:biotinylated BSA are listed in Table 7. A dilution of 1/4000 was required for the streptavidin-HRP to prevent background and non-specific binding. When the PDG:biotinylated BSA was added into the system, a strong signal was quickly generated at the anti-PDG antibody spot on the membrane.

Table 7. Activity of PDG:biotinylated BSA Antigen Preparation on Lateral Flow

PDG:Biotinylated BSA - Dilution	PDG:Biotinylated BSA - [μg/ml]	Streptavidin-HRP	Signal
0	0	1/100	blue background
0	0	1/2000	+ = NSB
0	0	1/4000	-
1/100	4.1	1/4000	+ @ 20 sec
1/10000	0.041	1/4000	+ @ 20 sec

The results for the PDG:streptavidin antigen are listed in Table 8. The biotinylated-HRP required a dilution of 1/5000 to prevent background and the PDG:streptavidin generated strong positive signal at dilutions of 1/1000-1/10,000.

Table 8. Activity of PDG:streptavidin Antigen Preparation on Lateral Flow

PDG:streptavidin - Dilution	PDG:streptavidin - [μg/ml]	Biotinylated HRP	Signal
0	0	1/1000	blue background
0	0	1/5000	-
1/1000	0.63	1/5000	+ @ 5 sec
1/5000	0.13	1/5000	+ @ 10 sec
1/10,000	0.063	1/5000	+ @ 20 sec - light

These experiments demonstrated the feasibility of using the biotin-streptavidin system in the lateral flow format to link PDG and HRP together. This is necessary for the final assay format. Experiments continued using these conjugates.

3.3.3. Feasibility Study for PDG Competitive Assay - PDG:Biotinylated BSA Antigen

Free PDG was used to compete with PDG:biotinylated BSA tagged antigen for binding to anti-PDG antibody on the membrane solid phase. Competition was determined by a decrease in HRP signal seen at the anti-PDG antibody spot on the membrane when free PDG was present.

Materials and Methods

Millipore ST membrane was spotted with 5 µl of Serotec anti-PDG antibody at 0.125, 0.25 and 0.5 mg/ml in PBS. To ensure an even protein coat on the membrane and improve the spot quality, the final protein concentration in all antibody solutions was brought to 0.5 mg/ml with BSA. PDG:biotinylated BSA at 1/100 and streptavidin-HRP at 1/4000 were mixed together in PB/BSA buffer. This mixture was incubated for 45 min. to allow the biotin and streptavidin to bind together. Free PDG was prepared at various concentrations in the same buffer matrix.

A multi-step assay protocol was performed in order to isolate each binding event on the membrane. The buffer "sample" containing the free PDG (100 µl) was migrated through the membrane for 10 min. This was followed by the PDG:biotinylated BSA-streptavidin-HRP mixture (100 µl) for 10 min. This two step process allowed the free PDG to bind to the anti-PDG antibody before tagged antigen was added to the system and should have given the best assay sensitivity. After all incubations, 20µl of TMB (KPL) substrate was applied directly to the membrane reaction sites.

This experiment was repeated using Millipore SX membrane which had a 3-4 fold increase in flow rate over ST membrane. This allowed more free PDG to migrate past the anti-PDG antibody on the membrane and compete with tagged antigen. The protocol was basically the same as described above only the PDG:biotinylated BSA was diluted 1/10,000 with streptavidin-HRP remaining at 1/4000 and Serotec antibody was spotted at 0.5 mg/ml.

Results and Discussion

The results for the first experiment using Millipore ST membrane are listed in Table 9. There was a decrease in assay signal when free PDG was migrated through the membrane. This competitive assay had a sensitivity of approximately 10 µg/ml. As the concentration of Serotec was decreased, the sensitivity of the competition increased. This was due to less antibody being available to bind tagged antigen after free PDG was bound.

Table 9. Competition of Free PDG and PDG:Biotinylated BSA Antigen on ST Membrane

Free [PDG]	Serotec (0.5 mg/ml)	Serotec (0.25 mg/ml)	Serotec (0.125 mg/ml)
SA @ 1/4000 only	-	-	-
0 µg/ml	+	+	+
1 µg/ml	+	+	+
10 µg/ml	+/-	-	-

The results for the fast flow Millipore SX membrane are listed in Table 10. The use of a faster flow membrane resulted in a significant increase in assay sensitivity to approximately 1 µg/ml. This was due to the fact that the fast flow membrane allowed exposure of the anti-PDG

antibody to a larger amount of free PDG by increasing the volume of free PDG that passed through the antibody spot.

Table 10. Competition of Free PDG and PDG:Biotinylated BSA Antigen on SX Membrane

Free [PDG]	Serotec = 0.5 mg/ml
0 μg/ml	+
0.1 μg/ml	+
1 μg/ml	+ lighter than 0 μg/ml
10 μg/ml	-

Both experimental results are significant and encouraging because they show that the PDG:biotinylated BSA tagged antigen will work in a competitive assay with an initial sensitivity of 1 μg/ml.

3.3.4. Feasibility Study for PDG Competitive Assay - PDG:Streptavidin Antigen

Free PDG was used to compete with PDG:streptavidin tagged antigen for binding to PDG antibody on the membrane solid phase. Competition was determined by a decrease in HRP signal seen at the anti-PDG antibody spot on the membrane when free PDG was present.

Materials and Methods

Millipore SX membrane was spotted with 5 μl of Serotec anti-PDG antibody at 0.5 mg/ml in PBS. PDG:streptavidin diluted 1/10,000 and biotinylated-HRP at 1/5000 were mixed together in PB/BSA buffer. This mixture was incubated for 45 min. to allow the streptavidin and biotin to bind together. Free PDG was prepared at various concentrations in the same buffer matrix.

Again, a multi-step assay protocol was performed to isolate each binding event on the membrane. The buffer "sample" containing free PDG (190 μl) was migrated through the membrane for 10 min. This was followed by the PDG:streptavidin-biotin-HRP mixture (190 μl) for 10 min. After all incubations, 50 μl of TMB (KPL) substrate was applied directly to the membrane reaction sites.

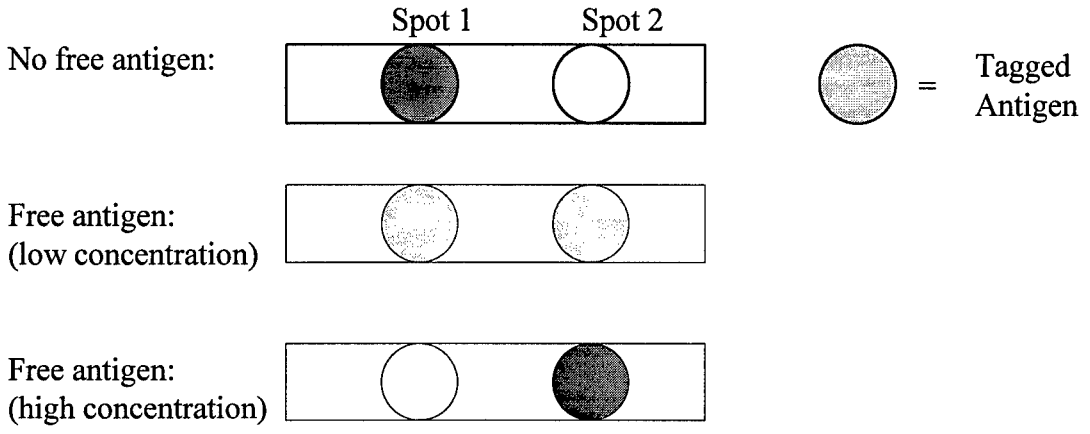
Results and Discussion

The results showed that signal at the anti-PDG spot was decreased at a free PDG concentration of 1 μg/ml. These results, similar to those obtained in the previous experiment, demonstrated that the avidin-biotin system could successfully be used to link PDG and HRP together in a competitive assay. They also showed an initial assay sensitivity of approximately 1 μg/ml.

These experiments used a more "traditional" competitive assay format where competition is indicated by a decrease in assay signal due to a decrease in the amount of tagged antigen present at the binding site. However, the assay format for this project is slightly different. The goal was to create an assay where there was an increase in signal due to the antigen competition. This was done by having multiple anti-PDG antibody spots on the membrane. The amount of tagged antigen was diluted to a point where it was completely bound by the antibody in the first spot. As free antigen was added into the system and bound to the first

spot, the tagged antigen migrated past the first spot and bound to antibody available at the second spot. This idea is depicted in figure 3.

Figure 3



The experimental focus for the project was to find the required concentration of the tagged antigen that allowed color to develop only at the first antibody spot.

3.3.5. PDG Competitive Assay Sensitivity Determination

The sensitivity of the PDG competitive assay using multiple antibody spots on the membrane solid phase was determined using PDG:streptavidin as the antigen combined with biotinylated HRP. A multi-step assay was performed to allow for the maximum competition to occur.

Materials and Methods

Affinity purified anti-PDG antibody (Serotec) was applied onto Millipore SR membrane at 0.58 mg/ml. Two 4 μl spots were placed on the membrane at 10 and 19 mm from the bottom. The membrane was dried at room temperature for 30 min. before use.

PDG:streptavidin was diluted into PB/BSA buffer containing biotinylated HRP at a 1:5000 dilution. The PDG-HRP mixture was incubated for 45 min. to allow the streptavidin and biotin to bind together. The dilutions were tested to determine what "titer" was required that allowed for color development at spot 1 but not spot 2. This dilution was determined to be 1/40,000 of the PDG:streptavidin combined with 1/5000 of the biotinylated HRP.

PDG was diluted in PB/BSA buffer at 0.01, 0.1, 1.0, 10, 20 μg/ml. This "sample" was applied to the glass fiber pad (150 μl) and allowed to migrate up the membrane for 20 min., after which 150 μl of the PDG-HRP conjugate mixture was migrated through the membrane for 20 min. Finally 150 μl of buffer only was migrated through the membrane to serve as a wash step (note: it was later determined that this wash step was not necessary). KPL TMB (50 μl) was applied directly to the membrane and allowed to react for 5 min. before color development was determined.

A modification of this protocol was also performed to make the assay a two-step procedure. The PDG-streptavidin plus the biotinylated HRP dilution was dried onto glass fiber pads (25 µl/pad) at 45°C for 30 min. This modification required the use of a lower dilution of the PDG:streptavidin antigen (1/5000) since a smaller volume was being used in the system. The test was run by adding sample to this glass fiber pad and letting the solution migrate up the membrane for 20 min. TMB substrate was then added to the membrane.

Results

The results for the PDG competitive assay are listed in Table 11. At a PDG concentration of 1 µg/ml signal is generated at spot 2. The results were similar for the two-step assay procedure using the dried glass fiber pads.

Table 11. PDG Competitive Assay Results

PDG [µg/ml]	Spot 1	Spot 2
0	+	-
0.01	+	-
0.1	+	-
1.0	+	+
10	-	+
20	-	+

Discussion

The sensitivity of the PDG assay was found to be lower than we targeted. The observations made during the feasibility study pointed to the lack of sensitivity being attributable to the anti-PDG antibody. The affinity of the Serotec antibody for PDG antigen did not seem to be as strong as the affinity of the anti-RabIgG antibody used in the RabIgG model studies.

We then focused efforts on determining the assay sensitivity of this platform using antibodies with known high affinities. Results proved that the assay sensitivity could be greatly improved over the sensitivity achieved with the PDG assay.

These results indicated that in order for a sensitive PDG assay to be developed, new antibodies need to be generated or further evaluation of the other commercially available antibodies needs to be performed.

The levels of PDG in women during the menstrual cycle varies from a low of 1-2 µg/ml to a high of 10-12 µg/ml. The most likely application for a PDG test device would be to measure the increase in PDG seen after ovulation. The sensitivity of the prototype test developed in this project is currently operating at this level of sensitivity for a clinically applicable PDG test device; however, the low sensitivity limit is too close to the bottom of the PDG range to construct a robust test. An additional increment of sensitivity is needed.

3.4. OTHER STUDIES

3.4.1. Urine Matrix Studies

Studies were conducted using a urine matrix instead of buffer. Male urine was used since female urine contains levels of PDG. The urine matrix performance was examined in the PDG and biotin assays.

The urine inhibited the binding of the PDG antigen to the anti-PDG antibody in both ELISA format and lateral flow which suggested a strong matrix effect or interference from some urine component. The effect could be eliminated by diluting the urine 1/10 into PB/BSA buffer. Lower dilutions of 1/4 and 1/2 also helped eliminate the negative effect. However, when the 1/4 dilution of urine was run in the competitive assay, no competition was seen. We believe this is due to the poor performance of the anti-PDG antibody used in this assay. This explanation is supported by results obtained in the development of the biotin assay.

3.4.2. Accelerated Stability Study

The PDG assay reagents were applied to membrane and glass fiber pads and placed on an accelerated stability study. Performance of the devices at the accelerated temperature of 45°C was compared to devices stored at RT.

Materials and Methods

Serotec anti-PDG antibody at 0.9 mg/ml in PBS was applied to Millipore SR membrane using the Biodot airbrush (4 μl). Two lines were applied at 10 and 19 mm from the bottom of the membrane. After drying at RT for 30 min. the membrane was applied onto a laminated card specifically used for lateral flow devices (G & L Precision Die Cutting Inc.) and cut into 6 mm wide strips.

The PDG antigen conjugate was prepared by mixing the biotinylated HRP at a 1/5000 and the streptavidin-PDG at 1/15,000 in PB/BSA. After a 45 min. incubation, 25 μl was dried onto glass fiber pads at 45°C for 30 min.

After drying, the pads and membranes were sealed in a foil pouch containing 1 g of desiccant and placed at RT and 45°C. Testing of the devices was done as a multi-step procedure. PDG was diluted into PB/BSA buffer and 150 μl migrated through the membrane for 20 min. The HRP-PDG glass fiber pad was then added to the device and 150 μl of PB/BSA buffer was used to migrate the conjugate through the membrane. After 20 min. TMB substrate from KPL (50 μl) was added to the membrane and color development noted.

Results

After 48 hours at the accelerated temperature both the conjugate pads and membrane showed signs of deterioration when tested. The conjugate pads stored at 45°C had no HRP activity when substrate was applied directly to the pads. The antibody on the membrane also showed some loss of activity compared to membrane stored at RT, however signal was still generated.

Discussion

The stability study has indicated that the HRP enzyme dried onto the glass fiber pad is very unstable. The stability should be improved by the use of protein and chemical stabilizers and by investigating various methods of drying the enzyme onto the pad. The material used for the HRP enzyme could also have an effect on the stability of the dried product.

The anti-PDG antibody was still active after 6 days at 45°C, which suggests that this component has good stability. The membrane had some slight color at the antibody lines for the membrane stored at the accelerated temperature which suggests that some change in the antibody is occurring.

3.5. OTHER FEASIBILITY STUDIES USING EAIC

3.5.1. Development of Biotin Assay

Since the desired sensitivity for the PDG assay was not achieved, we attempted to identify the cause of these results. We felt that the inferior affinity of the anti-PDG antibody caused the lack of sensitivity. We therefore attempted to develop an assay using known components of high affinity: biotin and streptavidin. Streptavidin has a known affinity for biotin of 10^{15}M-1. Biotinylated HRP was used along with free biotin to develop a competitive assay using EAIC.

Materials and Methods

Affinity purified streptavidin was prepared at 1 mg/ml in PBS and applied to a 5 X 25 mm strip of Millipore SR membrane by airbrush application (Biodot). The airbrush applied 2 µl per strip. The streptavidin was applied as two lines across the membrane at approximately 10 and 19 mm from the bottom. The membrane was dried at RT for 30 min.

Dilutions of biotinylated HRP were prepared in PB/BSA buffer. The biotinylated HRP was titered by applying 150 µl of solution to the glass fiber pad and allowing for migration through the membrane for 20 min. After 20 min., 50 µl of TMB substrate (KPL) was applied to the membrane and was allowed to react for 2 min. The color of each line was visually inspected. A dilution of 1/200,000 was required to allow color development at line 1 and no color development at line 2. This dilution was selected as the conjugate titer.

The biotinylated HRP titer dilution was then used to run the competitive assay. Free biotin was diluted 0, 25, 50, 100, 200 and 2000 ng/ml in PB/BSA buffer that also contained biotinylated HRP at 1/200,000. This reaction mixture was migrated through the streptavidin membrane as described in the titer step. After 20 min., 50 µl of TMB substrate was applied to the membrane and color was recorded after 2 min.

Results

The results for the competitive assay are listed in Table 12. A free biotin concentration of 25 ng/ml was able to prevent binding of a portion of the biotinylated HRP and allow for the conjugate to bind to line 2 and generate color. As the concentration of biotin increased (from 25 to 100 ng/ml) the intensity at line 2 increased. Concentrations of 200 ng/ml and higher completely blocked the biotinylated HRP from binding and generating color.

Table 12: Competitive Assay Results for Biotin Assay

Biotin [ng/ml]	Color @ Line 1	Color @ Line 2
0	+	-
25	+	+
50	+	+
100	+	+
200	-	-
2000	-	-

Discussion

The results of this experiment demonstrated that a supersensitive competitive assay could be performed using the EAIC technology. The assay sensitivity for this experiment is at least 25 ng/ml with a range of approximately 25 - 100 ng/ml. These results supported the idea that sensitivity is very dependent on the affinity of the antibody-antigen components. The results also indicate that the assay is semi-quantitative in that the color intensity at line 2 is dependent on concentration of free analyte. A color card could be generated comparing the intensity of the color at line 2 with analyte concentration in the sample.

3.5.1.1. Incorporation of Glucose Oxidase and TMB onto the Membrane Solid Phase

We next wanted to incorporate the glucose oxidase and TMB onto the membrane solid phase such that a 1-step assay could be performed. The use of glucose oxidase with glucose would generate the H_2O_2 necessary for the TMB-HRP reaction. The biotinylated HRP was dried onto the glass fiber pad.

Materials and Methods

A solution of 1 mg/ml TMB (Aldrich), 1 mg/ml streptavidin, 60 units/ml glucose oxidase in 10% ethanol and DI water was prepared. This solution could not be applied by the airbrush applicator due to oxidation of the TMB substrate (see page 9). Millipore SR membrane was spotted with the solution (4 µl) at 10 and 19 mm from the bottom of the membrane.

Biotinylated HRP was titered by dilution into PB/BSA buffer and 25 µl was dried onto the glass fiber pad at 45°C for 30 min. A dilution of 1/20,000 was selected as the conjugate titer. Free biotin was diluted in PB/BSA buffer. This "sample" was applied to the assembled membrane device (150 µl). Color developed immediately and final color development was recorded at 5 min.

Results

Results for the competitive assay are listed in Table 13. Biotin concentrations of 200 - 20,000 ng/ml caused color development at line 2. Again, color intensity at line 2 increased with concentration of biotin.

Table 13: Competitive Assay Results for Biotin Assay

Biotin [ng/ml]	Color @ Line 1	Color @ Line 2
0	+	-
20	+	-
200	+	+
2000	+	+
20,000	+	+

Discussion

These results show that assay sensitivity was still very good upon the incorporation of glucose oxidase, TMB and dried conjugate pads. This assay was performed as a one-step assay with "sample" application being the only step. We believe the assay sensitivity could be improved to 20 ng/ml with further optimization of the assay components. The dynamic range of the assay was greatly expanded from the previous biotin experiment (20-20,000 ng/ml vs. 25-100 ng/ml). These results indicate that the assay performance with respect to both sensitivity and range is very dependent on the EAIC components used.

3.5.1.2. Urine Matrix Study

The competitive biotin assay was performed using urine as the sample matrix. The glucose oxidase and TMB were applied to the membrane solid phase. Results were compared to the performance results seen in the previous experiment that used buffer.

Materials and Methods

The streptavidin membrane was prepared as in the previous experiment and the biotinylated HRP was diluted into urine containing levels of free biotin. This mixture (150 µl) was migrated through the membrane. Color developed immediately and final color development was recorded at 5 min.

Results and Discussion

The results obtained using urine as the sample matrix were identical to those obtained with the buffer experiments. The sensitivity of the assays were approximately 200 ng/ml essentially as observed in the survey listed in Table 13.

These results indicate that a urine matrix can be used without adverse effects on assay sensitivity and EAIC component performance.

3.5.2. Development of Anti-FITC Assay

We wanted to show that a sensitive competitive immunoassay could be developed using the EAIC if high affinity antibodies were used. FITC was selected as a model system due to availability of antibodies with high affinity for FITC.

Materials and Methods

Millipore SR membrane was spotted with anti-FITC monoclonal antibody. Spot 1 was 5 µl and spot 2 was 2.5 µl and they were spotted 10 and 19 mm from the bottom of the membrane. The membrane was dried at room temperature for 30 min.

An titer experiment using HRP labeled FITC diluted in PB/BSA determined that a dilution of conjugate of 1/200,000 allowed color development at spot 1 but not spot 2. Conjugate pads were prepared by drying 25 µl of 1/200,000 HRP-FITC at 45°C for 30 min.

Free FITC was prepared as a 2 mg/ml stock in DMSO. This stock solution was then diluted into PB/BSA at 20, 100, 200 and 2000 ng/ml. The "sample" was applied to the conjugate pads (150 µl) and allowed to migrate through the membrane for 10 min. TMB substrate from KPL (50 µl) was than added to the membrane and color development was recorded.

Table 14: FITC Competitive Assay Results

FITC [ng/ml]	Spot 1	Spot 2
0	+	-
20	+	-
100	+	+
200	+	+
2000	-	-

Results

The results for the competitive assay are listed in Table 14. Color developed at spot 2 at FITC concentrations of 100-200 ng/ml.

Discussion

This experiment demonstrated that a sensitive EAIC competitive immunoassay could be developed by using high affinity antibodies. The sensitivity of this assay was at least 100 ng/ml and we believe it can be optimized to increase sensitivity even further. Unlike the biotin assay, the dynamic range of this assay was very narrow. The dynamic range of this assay could be expanded by increasing the number of antibody "zones" on the membrane or by increasing concentration of antibody at the selected zones.

3.5.3. Development of Specific Antibody Test

A mouse model was used to develop a test that would measure specific antibody. We wanted to determine what level of an antigenic specific antibody could be detected in the presence of the entire IgG population in a serum sample. Human IgG was used as the antigen and mouse anti-human IgG was used as the specific antibody. This antibody was added into mouse serum at various concentrations. The lowest level of mouse anti-human IgG that could be detected bound to the human IgG on the membrane was determined to be the sensitivity of the assay.

The bound mouse anti-human IgG was detected by goat anti-mouse IgG-HRP and the EAIC technology.

Materials and Methods

Human IgG at 1 mg/ml in PBS was sprayed (2 μl) onto Millipore SR membrane using the Biodot airbrush. The membrane was dried at RT for 30 min. before use.

Goat anti-mouse IgG-HRP diluted 1/5000 in PB/BSA buffer was applied to glass fiber pads (25 μl) and dried at 45°C for 30 min.

The assay was run as a multi-step procedure. The first step consisted of migrating 150 μl of mouse serum spiked with the mouse anti-human IgG through the membrane. Unspiked mouse serum was run as a negative control. After a 10 min. incubation, the dried conjugate pads were added to the membrane and 150 μl of 50 mM phosphate was used to migrate the goat anti-mouse IgG-HRP through the membrane (step 2). After a 10 min. incubation, 50 μl of TMB (KPL) substrate was added to the membrane.

A second experiment was run using mouse anti-human IgG spiked into PB/BSA buffer instead of mouse serum. The assay was performed as above except steps 1 and 2 were combined.

Results

A concentration of 200 ng/ml of specific antibody in the mouse serum was detected by EAIC. The negative control of the mouse serum alone did not generate any positive signal on the membrane.

A sensitivity of 20 ng/ml was demonstrated when the assay was performed in buffer.

Discussion

These results are very significant in that they demonstrate the feasibility of an assay to determine the level of specific antibody in a complex sample matrix of serum which contains high levels of non-specific antibody. It also indicates that the EAIC technology is compatible in a serum matrix. This assay was previously carried out using goat anti-mouse conjugated to colloidal gold as the detection reagent. In this system, the lowest amount of specific antibody that could be detected was 50 μg/ml. Thus, these results confirm earlier studies that indicate the EAIC technology is much more sensitive than colored particle-based lateral flows.

4. CONCLUSIONS

The key technical objectives completed for the Phase I project and their implications are as follows:

- Technical feasibility of enzyme amplified immunochromatography (EAIC) was demonstrated. This technology employed two enzymes, glucose oxidase and horseradish peroxidase, to generate a visually detectable color at the site of a specific antibody-mediated binding event. Previous studies have demonstrated that EAIC creates a 10 to 100 fold increase in visual sensitivity over the use of dyed particles (3).

- PDG antigen was successfully coupled to streptavidin and biotinylated BSA. These conjugated molecules remained biologically active and were used to link the HRP enzyme to the PDG antigen. This technical achievement eliminated the step of attaching the antigen and enzyme onto a "carrier" latex particle. This simplified the development of the test and allowed flexible concentration adjustments of these two critical components.
- A sensitive competitive immunoassay was developed for the urinary metabolite of progesterone, PDG. The sensitivity of this assay was 1 μg/ml with a dynamic range of 1 - 20 μg/ml. These levels are clinically significant for measuring this hormone metabolite in female urine to monitor the phases of the menstrual cycle. The test requires a minimal amount of sample preparation and is simple and rapid to perform. A two step assay was performed by adding a clinical sample followed by TMB substrate. This can be reduced to a one-step assay by applying the TMB onto the membrane solid phase.
- The results for initial accelerated stability studies indicated that the glucose oxidase and TMB components of the assay were very stable at the accelerated temperature of 45°C for 7 to 10 days. The stability of the PDG test device was found to be unstable after 3 days at 45°C. Analysis of the individual components in this study indicated that the HRP enzyme lost activity at the accelerated temperature. Modifications to the buffer formulation for this component will greatly improve the stability of the enzyme.
- The sensitivity of the PDG assay should be improved by use of anti-PDG antibodies which retain high binding affinities following their immobilization on the strip test membrane. This statement is strongly supported by the fact that other antibodies and model systems described below have demonstrated sensitivities of 20 to 200 ng/ml using the EAIC technology.
- Feasibility studies using streptavidin as the model capturing agent and biotin as the model antigen resulted in a one-step, 20 minute test with a sensitivity of 200 ng/ml. These same reagents were used to develop a rapid two step test with a 20 ng/ml sensitivity. We believe the increased sensitivity seen with this assay compared to the PDG assay was due to the high affinity of streptavidin for biotin.
- An EAIC test was shown to be feasible using anti-FITC antibody and FITC-HRP as the tagged antigen. This 10 minute test had a sensitivity of at least 100 ng/ml. The anti-FITC antibody was known to have high affinity for the FITC molecule.
- A sandwich assay was demonstrated which detected 200 ng/ml of specific antibody in whole serum, using mouse serum as the model. These results indicate that the EAIC technology can be applied to a multiple antibody format with a high level of sensitivity. It also demonstrates the performance of the assay in the complex matrix of serum.

5. REFERENCES

1. Adlercreutz H., Brown J., Collins W., Goebelsman U., Kellie A., Campbell H., Spieler J. And Braissand G.: The Measurement of Urinary Steroid Glucuronides as Indices of the Fertile Period in Women. *J. Steroid Biochem.* **17** (1982) 695-702.

2. Bangs L.: New Developments in Particle Based Tests and Immunoassays. Presented at "the Latex Course", Diagnostic Applications of Latex Technology", Indianapolis IN, May 6-8, 1992

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6. APPENDICIES

NONE

7. PERSONNEL RECEIVING PAY FOR THE NEGOTIATED EFFORT

Dr. Michael Brinkley - Principal Investigator
Dr. Geoffrey Seaman - Coinvestigator
Dr. Paul Hemmes - Consultant
Ms. Gail Shannon - Technician
Mr. Mark Jackson - Technician

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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